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Neurotransmitter Imbalance in the Brain and Alzheimer's Disease Pathology

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Abstract.

Background: Cholinesterase inhibitors represent three of the four treatments for Alzheimer's disease (AD), and target the pathological reduction of acetylcholine levels. Here we aimed to study the role of other neurotransmitter pathways in AD pathology.

Objective: This study aimed to determine associations between AD pathology at both symptomatic and asymptomatic stages of disease progression, and the metabolism of a range of non-cholinergic neurotransmitters.

Methods: Tissue samples were obtained from three groups, controls, AD, and 'asymptomatic AD' (ASYMAD), i.e., cognitively normal individuals that had significant AD neuropathology. Three brain areas were studied, the middle frontal gyrus (MFG), the inferior temporal gyrus (ITG), and the cerebellum.

Results: 12 of 15 metabolites involved in neurotransmitter metabolism were shown to be associated with AD pathology. Decreases in dopamine were most pronounced in the MFG with lower levels seen in the ASYMAD group compared to control ($FC = 0.78, p = 2.9 \times 10^{-2}$). In the ITG significant changes were seen in GABAergic and serotonin metabolism between control and AD patients; however, these changes were not seen between control and ASYMAD individuals.

Conclusion: These results indicate that dopamine could be depleted in brains with AD pathology but intact cognition, while an imbalance of several neurotransmitters is evident in the brains of AD patients.

Keywords: Asymptomatic Alzheimer's disease, brain, metabolomics, neurotransmitters

INTRODUCTION

Dementia is a devastating illness for both patients and their families, with Alzheimer's disease (AD) estimated to account for up to 80% of total dementia cases. The 'World Alzheimer's Report 2015'

estimates that there are approximately 46 million AD patients worldwide, with this number expected to rise to over 130 million by the middle of the century [1]. As well as a significant human cost, AD also represents a major financial burden with worldwide costs related to AD expected to reach \$1 trillion dollars in 2018 [1].

Cholinesterase inhibitors make up three of the four approved AD treatments (donepezil, rivastigmine, and galantamine), making inhibition of acetylcholinesterase the leading therapeutic strategy for the treatment of AD symptoms [2, 3]. There

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is a significant body of literature suggesting that the cognitive deficits associated with AD are the result of lower levels of acetylcholine in the brain resulting from dysfunction of cholinergic neurons [4–6]. The role of non-cholinergic neurotransmitter systems in AD pathogenesis has received less attention. While levels of non-cholinergic neurotransmitters in the brain have been associated with AD pathology [7–11], their role in mediating the onset of symptoms is less well understood. In this study, we analyzed data from a non-targeted metabolomics platform to compare differences in neurotransmitters and neurotransmitter-associated metabolite levels in brain tissue samples from the autopsy cohort of the Baltimore Longitudinal Study of Aging (BLSA). We studied three groups of BLSA participants, AD patients, cognitively normal controls, and ‘asymptomatic AD’ (ASYMAD), i.e., individuals with significant AD neuropathology at death but with no evidence of cognitive impairment during life. We studied three distinct brain regions in these individuals that are differentially affected pathologically: the inferior temporal gyrus (ITG) which is vulnerable to neurofibrillary tau tangles, the middle frontal gyrus (MFG) which is susceptible to the accumulation amyloid- β plaques, and the cerebellum which is resistant to classical AD pathology [12]. This study aimed to determine if non-cholinergic neurotransmission might also be dysregulated in the pathology of AD. We also wanted to see if the metabolism of neurotransmitters was being modulated differently in different brain regions with differing levels of amyloid- β and tau.

METHODS

Chemicals and reagents

All solvents, water, acetonitrile, methanol, ammonium formate, formic acid, and methyl tertiary butyl ether, were LC-MS grade purchased from Sigma-Aldrich. Internal standards L-serine $^{13}\text{C}_3^{15}\text{N}$ (95% pure) and L-valine $^{13}\text{C}_5^{15}\text{N}$ (95% pure) were purchased from Sigma-Aldrich. In vial dual extractions were performed in amber glass HPLC vials with 400 μl fixed inserts (Chromacol: Welwyn Garden City, UK).

Sample information

The BLSA is a prospective, ongoing cohort study of community-dwelling volunteer participants in

Baltimore begun in 1958. As such, it is among the largest and longest-running longitudinal studies of aging in the United States [13, 14]. In general, at the time of entry into the study, participants had no physical or cognitive impairment. Detailed examinations, including neuropsychological assessments and neurological, laboratory, and radiological evaluations, were conducted every 2 years. Since 2003, participants older than 80 years have received yearly assessments. Written informed consent was obtained at each visit, and the study was approved by the local Institutional Review Board and the National Institute on Aging. After each visit, cognitive status was considered relying on information from neuropsychological tests as well as clinical data as described previously [15]. Diagnoses of dementia and AD were based on DSM-III-R [16] and the NINCDS-ADRDA criteria [17], respectively.

Brain tissue samples were collected through the autopsy sample of the BLSA. The autopsy program of the BLSA was initiated in 1986. We have previously described the study protocol in detail. Briefly, the mean age at death in the autopsy sample is 88.3 ± 7.3 years (range 69.3–103.2), and the mean interval between the last evaluation and death is 8.7 ± 6.7 months [18]. As reported previously, the autopsy subsample is not significantly different from the BLSA cohort as a whole in terms of the rates of dementia and clinical stroke [19]. Table 1 describes the demographic characteristics of the participants whose brain tissue samples were used in this study, with the same sample set having been used previously in the study of fatty acid metabolism described in Snowden et al. [20].

Sample preparation and LC-MS analysis

Thirteen of the fifteen metabolites described in this paper (tyrosine, L-DOPA, dopamine, aminobutanol, arginine, aspartate, GABA, glutamate, glutamine, guanidinobutanoate, glycine, guanosine, and ornithine) were measured on an in-house platform that is described in detail in Ebshiana et al. [21]. Briefly 20 μl of methanol and 5 μl of internal standard solution (2.5 mM L-serine $^{13}\text{C}_3^{15}\text{N}$ and L-valine $^{13}\text{C}_5^{15}\text{N}$ in 4:1 methanol:water) was added per milligram of tissue prior to homogenization. Tissue was homogenized using a 4-mm stainless steel ball bearing and Tissuelyzer (Qiagen) in 10 cycles of 30 s at 25 Hz. Subsequently 50 μl of homogenate was transferred to a HPLC vial (400 μl fixed insert), to which 10 μl water and 250 μl of methyl-tertiary butyl ether

Table 1
Clinical characteristics of study participants

	Control	Asymptomatic AD	Alzheimer's Disease
Participants	14	15	14
Sex (female/male)	4/10	5/10	7/7
Age at death (y)*	82.6 ± 11.0 (64.2–99.2)	89.2 ± 7.9 (71.9–96.4)	87.9 ± 8.9 (62.9–98.7)
MMSE [†]	27.8 ± 2.4	29.0 ± 0.9*	23.0 ± 6.9*
PMI (h) [‡]	16.9 ± 6.4 (7.0–28.0)	14.8 ± 8.1 (2.0–33.0)	14.7 ± 6.0 (3.0–23.0)
Cholinesterase/NMDA agonist usage	0/0	0/0	2/0

*Values are reported as the mean ± standard deviation, and range, [†]values are reported as the mean ± standard deviation; [‡]values are reported as the mean ± standard deviation, and range. MMSE; Mini-Mental State Examination, NMDA, N-methyl-D-aspartate, PMI, postmortem interval.

were added prior to vortexing at room temperature for 1 h. Following this, 40 µl of water containing 0.15 mM ammonium formate was added, and samples were then spun at 2500 × g for 30 min at 4°C. Prior to analysis, samples were stored at –20°C with samples and QCs stored for no longer than 7 days prior to analysis.

Samples were analyzed on a Waters Acquity ultra performance liquid chromatography (UPLC) system coupled to a Waters premier quadrupole time-of-flight (Q-ToF) mass spectrometer (Waters, Millford, MA, USA). Briefly, 5 µl of the aqueous phase was injected on a Merck Sequant Zic-HILIC column (150 × 4.6 mm, 5 µm particle size) coupled to a Merck Sequant guard column (20 × 2.1 mm). The gradient was performed over 40 min at room temperature with a flow rate of 0.3 ml/min using 0.1% formic acid in water (mobile phase A) and 0.1% formic acid in acetonitrile (mobile phase B). The gradient started at 80% mobile phase B, followed by a linear reduction to 20% B at 30 min followed by 10 min re-equilibration at the initial conditions. Mass spectral data was acquired between 75–1000 daltons, and data was collected in centroid mode with an acquisition time of 0.1 s per scan.

Additional metabolite analysis

Data on the abundance of serotonin and tryptophan were acquired using the Biocrates platform. To extract metabolites from brain tissue, samples were homogenized using Precellys[®] with ethanol phosphate buffer. Samples were then centrifuged and the supernatant was used for analysis. The fully automated assay was based on liquid chromatography-tandem mass spectrometry (LC-MS/MS; amino acids) using a SCIEX 4000 QTrap[®] mass spectrometer (SCIEX, Darmstadt, Germany) with electrospray ionization. Brain tissue concen-

tration was absolute concentration expressed as nmol/mg tissue.

Statistical methods and pathway mapping

To compare the abundance of neurotransmitters and their associated metabolites among the three groups (CN, ASYMA, and AD), we used generalized linear models (GLM) performed in 'R' (version 3.4.2), with all the calculated models corrected for both age and sex. With the three patient groups potentially representing distinct biological phenotypes, we decided to focus on binary comparisons between groups. The relationship of metabolite abundance to measures of cognitive performance was assessed by comparing the metabolite abundance with measures of cognition, the Mini-Mental State Examination (MMSE), Boston naming test, and Benton visual retention score, this was done again using GLM performed in 'R' (version 3.4.2).

Pathway mapping was performed in Cytoscape (version 3.4.0) with the architecture of the displayed pathways determined by metabolic interactions defined in the Kyoto Encyclopedia of Genes and Genomes (KEGG). Within the network, node size is directly proportional to the fold change in metabolite abundance between control and AD groups, with edge thickness directly proportional to the partial correlation of the two nodes it is connecting. The partial correlation coefficients were calculated using Pearson's correlation to compare the abundance of the two metabolites in all samples in the given brain region.

RESULTS

Analysis of control versus ASYMA groups

The abundance of all fifteen metabolites were compared between control and ASYMA individuals

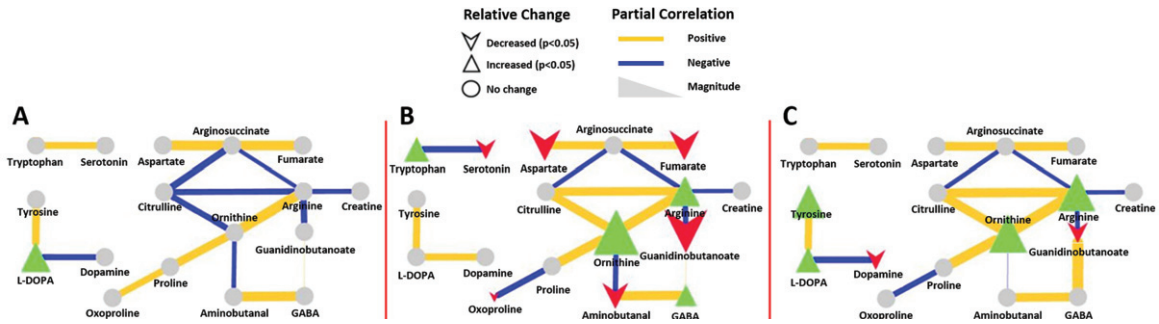


Fig. 1. Showing pathway analysis of the association of neurotransmitter metabolism to Alzheimer's disease in human brain using the comparison of control and AD individuals. Metabolites significantly increased in abundance ($p < 0.05$) and shown as green triangles and metabolites significantly decreased in abundance ($p < 0.05$) and shown as red chevrons with the size representing the magnitude of the change. Grey circles represent metabolites that were not significantly associated with disease. A) shifts observed in the cerebellum, B) shifts observed in the inferior temporal gyrus, C) shifts observed in the middle frontal gyrus.

(Fig. 1, Table 2, Supplementary Figure 1). The only neurotransmitter that had a significantly altered abundance was dopamine which was decreased in the MFG ($FC = 0.78$, $p = 2.9 \times 10^{-2}$). In the ITG, aminobutanol, aspartate, and guanidine, which are all involved in the metabolism of GABA, were decreased in abundance in the ASYMD patients ($FC = 0.86$ & $p = 4.3 \times 10^{-2}$, $FC = 0.83$ & $p = 2.7 \times 10^{-2}$, and $FC = 0.72$ & $p = 3.3 \times 10^{-2}$, respectively) (Fig. 1, Table 2, Supplementary Figure 1). All other measured metabolites showed no significant difference between control and ASYMD groups in any of the brain regions analyzed.

Analysis of control versus AD groups

In the comparison of control versus AD groups, changes were observed mainly in the ITG. In the ITG, excitatory neurotransmitters glutamate and aspartate exhibited a lower abundance ($p < 0.05$) in AD patients. Also, in the ITG, inhibitory neurotransmitters glycine and serotonin were decreased, while GABA was increased ($p < 0.05$). A number of neurotransmitter precursors were also increased: ornithine, arginine, and tryptophan (all $p < 0.05$), while guanidobutanoate, guanosine, and aminobutanol were all significantly decreased (all $p < 0.05$) in the ITG of AD patients (Fig. 1, Table 2, Supplementary Figure 1). In the MFG, dopamine precursors L-DOPA and tyrosine were the only metabolites to be increased with disease. An increase in L-DOPA was the only significant difference observed in the cerebellum (Fig. 1, Table 2, Supplementary Figure 1).

Analysis of ASYMD versus AD groups

In the comparison of AD versus ASYMD, two changes were observed in the MFG: GABA was increased ($FC = 1.23$, $p = 1.3 \times 10^{-2}$) and guanidobutanoate was decreased ($FC = 0.84$, $p = 1.9 \times 10^{-2}$). In the ITG, guanidobutanoate and glycine were decreased ($FC = 0.69$, $p = 1.2 \times 10^{-4}$, $FC = 0.74$, $p = 5.0 \times 10^{-3}$, respectively) (Table 2, Supplementary Figure 1). There were no other significant differences observed between ASYMD and AD groups in any of the analyzed brain regions.

Correlation of metabolite abundance and cognitive performance

Analysis to investigate the relationship between metabolite abundance and cognitive performance showed that dopamine, tryptophan, aspartate, aminobutanol, GABA, and guanidobutanoate positively correlated with MMSE ($p < 0.05$) in all regions, while tyrosine and arginine were negatively correlated with MMSE ($p < 0.05$) (Supplementary Table 1). When the analysis was performed using Benton's visual retention index as the measure of cognitive performance, tyrosine, L-DOPA, tryptophan, arginine, and GABA positively correlated with Benton's visual retention index ($p < 0.05$) (Supplementary Table 1), while dopamine, aminobutanol, aspartate, and guanidobutanoate were negatively correlated ($p < 0.05$). However, only guanidobutanoate and tryptophan correlated with the Boston naming score ($p < 0.05$) (Supplementary Table 1).

Table 2
Relative changes in abundance of 15 metabolites associated with neurotransmitter metabolism between all three diagnostic groups in individual brain regions

	Control versus asymptomatic						Control versus dementia						Asymptomatic versus dementia					
	Cerebellum			MFG (Aβ)			Cerebellum			MFG (Aβ)			Cerebellum			MFG (Aβ)		
	FC*	p [†]	ITG (tau)	FC*	p [†]	ITG (tau)	FC*	p [†]	ITG (tau)	FC*	p [†]	ITG (tau)	FC*	p [†]	ITG (tau)	FC*	p [†]	ITG (tau)
Tyrosine	1.19	0.229	1.21	0.151	1.39	0.57	1.17	0.251	1.26	0.099	1.49	0.044	0.99	0.925	1.04	0.772	1.07	0.647
L-DOPA	1.21	0.120	1.06	0.539	1.12	0.369	1.35	0.018	1.06	0.454	1.33	0.016	1.12	0.163	1.00	0.952	1.19	0.125
Dopamine	0.98	0.896	0.97	0.830	0.78	0.015	0.82	0.183	0.88	0.287	0.78	0.029	0.83	0.228	0.90	0.524	0.99	0.955
Tryptophan	0.99	0.935	1.36	0.085	1.24	0.177	1.72	0.359	1.45	0.043	1.40	0.124	1.18	0.302	1.06	0.756	1.12	0.531
Serotonin	N/M	N/M	1.07	0.378	0.87	0.384	0.89	0.722	0.73	0.034	0.99	0.177	0.83	0.110	0.69	0.083	1.13	0.492
Aminobutanol	1.01	0.779	0.86	0.043	0.93	0.262	0.94	0.304	0.76	0.009	0.93	0.161	0.93	0.256	0.89	0.154	1.00	0.951
Arginine	0.96	0.794	1.38	0.102	1.48	0.075	1.10	0.514	1.59	0.013	1.45	0.055	1.14	0.425	1.15	0.401	0.99	0.936
Aspartate	1.00	0.989	0.83	0.027	0.94	0.439	0.95	0.485	0.71	0.009	0.92	0.159	0.95	0.457	0.86	0.100	0.97	0.746
GABA	1.05	0.575	1.06	0.112	1.01	0.339	1.24	0.142	1.13	0.005	1.23	0.035	1.07	0.315	1.05	0.150	1.23	0.013
Glutamate	1.05	0.371	0.96	0.460	0.98	0.590	1.04	0.364	0.90	0.022	0.98	0.603	0.99	0.806	0.93	0.220	1.00	0.906
Glutamine	1.04	0.680	0.98	0.801	1.05	0.662	1.05	0.577	0.91	0.111	1.04	0.643	1.00	0.974	0.92	0.310	0.99	0.944
Guanidinobutanoate	0.91	0.939	0.88	0.389	1.04	0.179	0.82	0.795	0.61	0.000	0.81	0.013	0.90	0.892	0.69	0.000	0.78	0.083
Glycine	0.99	0.326	1.11	0.129	1.14	0.559	0.97	0.750	0.83	0.018	0.95	0.260	0.99	0.325	0.74	0.005	0.84	0.019
Guanosine	0.98	0.941	0.72	0.033	0.87	0.388	1.16	0.442	0.64	0.012	0.70	0.035	1.18	0.476	0.89	0.488	0.80	0.250
Ornithine	0.94	0.593	1.27	0.132	1.41	0.078	1.04	0.692	1.41	0.014	1.41	0.045	1.11	0.376	1.11	0.454	1.00	0.997

*Mean fold change relative to controls; †p-value calculated using generalized linear models; ‡ mean fold change relative to asymptomatic. DOPA, dihydroxy-phenylalanine; GABA; gamma-aminobutanoate; ITG, inferior temporal gyrus; MFG, medial frontal gyrus; N/M, not measured.

DISCUSSION

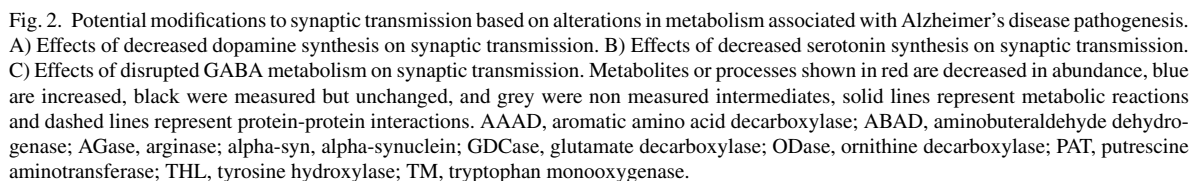
The metabolism of neurotransmitters is an important consideration in the pathology of all neurological diseases. In this study, we measured the metabolism of three key excitatory neurotransmitters dopamine, glutamate, and aspartate, as well as three inhibitory neurotransmitters, serotonin, glycine, and GABA. We tested to see if any observed changes in the abundance of neurotransmitters was associated with the asymptomatic AD group, and to determine if specific brain regions exhibited unique differences in neurotransmitter metabolism.

Dopaminergic depletion in brains with AD neuropathology and normal cognition

Dopamine is a catecholamine neurotransmitter [22, 23], which plays several important roles in the brain acting via four distinct pathways, the mesolimbic, mesocortical, nigrostriatal, and tuberoinfundibular pathways. These pathways are responsible for regulating mood, and aiding cognitive and motor function. Impairment of this system potentially causes depression [24], memory loss [25], and impaired motor control observed in patients with AD.

Dopamine does not cross the blood-brain barrier and is synthesized in two steps from the essential amino acid tyrosine, with the initial conversion of tyrosine to L-DOPA catalyzed by tyrosine hydroxylase (TH) with the subsequent conversion of L-DOPA to dopamine catalyzed by aromatic amino acid decarboxylase (AAAD). In the MFG, a comparable reduction in dopamine is observed in both the asymptomatic and symptomatic AD patients (Fig. 2, Supplementary Figure 1). In the MFG, tyrosine and L-DOPA, the precursors of dopamine, are elevated, with a reduction in the abundance of dopamine also observed, suggesting a decrease in the abundance or activity of TH and AAAD.

The activity of both TH and AAAD is inhibited by alpha-synuclein [26], and has been implicated in the pathology of several neurodegenerative diseases including Parkinson's disease [27], AD [28], and Lewy bodies dementia [27–29]. Studies have also shown that soluble intraneuronal alpha-synuclein, in the absence of Lewy body pathology is increased in abundance by up to two-fold in the brain of AD patients [30, 31]. This suggests a plausible molecular mechanism by which alpha-synuclein may modulate brain dopamine concentrations in AD. Reduced dopamine could reduce the amount of the



Neurotransmission inhibition in the inferior temporal gyrus in AD

brains of AD patients [30, 31], has been shown to inhibit the action of AAAD [26], suggesting a potential co-mechanism leading to decreased serotonin synthesis as observed in this study. While serotonin levels in the AD brain are marginally significant, it is interesting as recent reports have shown that antidepressants such as Trazodone, a serotonin antagonist, could help maintain neural integrity [32, 33].

Glutamate was also reduced in the ITG of AD patients; this was surprising as glutamate activation of N-methyl-D-aspartate (NMDA) on the post synaptic neuron and its excitotoxicity have long been implicated in the pathology of AD [34–36]. The impairment of the glutaminergic system in the brain leads to impairment of a range of neurological functions, including fast excitatory neurotransmission [37], memory and learning [38], and long term potentiation [39–41]. The role of glutamate in AD

is well known. Memantine, an NMDA antagonist, is used to treat moderate to severe AD, and has been shown to have affinity for dopamine receptors which is interesting in light of this report's findings [42].

In this study, GABA is increased in abundance but no change is seen in glutamate levels apart from a modest shift observed in the ITG. While increased GABA production could still be coming at least in part from glutamate, the changes observed in other metabolites associated with GABA metabolism mean that the alterations may arise from multiple pathways (Fig. 2). GABA is the chief inhibitory neurotransmitter in the mammalian nervous system [43, 44]. It does not cross the blood-brain barrier [45] and in the brain is predominantly synthesized from the non-essential amino acid glutamate by the action of glutamate decarboxylase under standard physiological conditions [46]. However, GABA can be synthesized via several pathways from selection precursors, including aminobutanol by aminobutyraldehyde dehydrogenase [47], succinate semialdehyde by aminobutyrate aminotransferase [48], and guanidinobutanoate by guanidinobutyrase (Supplementary Figure 2). Two alternative GABA synthetic pathways, both of which start from the urea cycle, have intermediates that are significantly reduced in abundance (Supplementary Figure 2) suggesting that they may play a role in the dysregulation of GABA metabolism.

Regardless of the synthetic source of the increased abundance of GABA, this combined with the reduction in glutamate in the ITG (Table 2) can produce a reduction in their ratio, leading to an inhibitory environment and a reduction in the transmission of action potentials. When GABA is released into the synaptic cleft it binds to a range of transmembrane receptors on both the pre and post-synaptic neurons leading to the opening of ion channels allowing the negatively charged chloride ions to enter and positively charged potassium ions to escape the neuron (Fig. 2) [49]. This shift leads to loss of the transmembrane potential and hyperpolarization of the cell membrane, inhibiting action potentials produced by excitatory neurotransmitters like glutamate.

Strengths and limitations

The main strengths of this study are the use of samples from the well-characterized BLSA, which has serial cognitive assessments and detailed neuropathological examination postmortem. This allows the identification and inclusion of the asymptomatic

group enabling us to relate the observed shifts in metabolism with the severity of pathology as well as the expression of symptoms. The analysis of the three brain regions representing areas that are differentially susceptible to amyloid and tau pathology to determine how each of these proteins could be associated with neurotransmitter metabolism independently.

The main limitation of this study is its relatively small sample size, which means that our findings need independent validation. However, there are few cohorts with the extensive longitudinal cognitive assessment and postmortem neurological examination means that identifying a sample set that includes asymptomatic individuals is difficult.

Another possible limitation of this work is that neurotransmitters can be unstable and likely to break down after death, hence postmortem interval (PMI) (time between death and sample collection) is an important consideration. The postmortem interval in the control group is not significantly higher compared to the asymptomatic and AD groups (Table 1). As PMI in each of the groups is the same, degradation of these unstable metabolites should be comparable, meaning that the molecular relative abundances between groups should be similar.

Conclusion

In conclusion, our results showed decreased levels in dopamine in brains with pathology from patients who exhibited no cognitive deficit during life. In AD brains, neurotransmitter dysregulation was observed in several pathways and correlated with cognitive tests in AD. Combined therapeutic approaches that consider the GABAergic and serotonergic pathways might be useful as adjunctive treatments in AD.

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Authors' disclosures available online (<https://www.j-alz.com/manuscript-disclosures/19-0577r2>).

SUPPLEMENTARY MATERIAL

The supplementary material is available in the electronic version of this article: <http://dx.doi.org/10.3233/JAD-190577>.

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